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We have evidence that the nuclear matrix protein HET might represent an important tumor suppressor gene in human breast cancer: 1. Overexpression of HET inhibited growth. 2. It was negatively associated with S-phase fraction in breast tumors, and 16% of breast tumors did not express HET. 3. Western Blot analysis in breast tumors led to the detection of smaller products, presumably representing truncated HET proteins. 4. HET maps to a locus on chromosome 19p13 where we detected an unusually high rate of loss of heterozygozity. In the first specific aim we will directly answer whether HET is the tumor suppressor gene by performing additional LOH analysis and mutational analysis of HET in breast cancer cell lines as well as in tumors. In the second specific aim we will perform functional analysis of discovered HET mutations in breast cancer cell lines. If HET is the tumor suppressor gene at 19p13, this will have a direct impact on our mechanistic understanding of tumor suppressor genes in breast cancer, as this region has an extremely high LOH rate in human breast cancer. If our hypothesis is true, then mutational analysis of HET could become a very informative tool for breast cancer prognosis and therapy.

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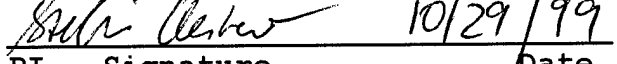

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INTRODUCTION

As presented in our original grant proposal, we have evidence that the nuclear matrix protein HET might represent an important tumor suppressor gene in human breast cancer: 1. Overexpression of HET inhibited growth. 2. It was negatively associated with S-phase fraction in breast tumors, and 16% of breast tumors did not express HET. 3. Western Blot analysis in breast tumors led to the detection of smaller products, presumably representing truncated HET proteins. 4. HET maps to a locus on chromosome 19p13 where we detected an unusually high rate of loss of heterozygozity. Excited by this preliminary observation, we proposed to examine whether HET is the tumor suppressor gene at 19p13. In the first specific aim we will directly answer whether HET is the tumor suppressor gene by performing additional LOH analysis and mutational analysis of HET in breast cancer cell lines as well as in tumors. In the second specific aim we will perform functional analysis of discovered HET mutations in breast cancer cell lines. If HET is the tumor suppressor gene at 19p13, this will have a direct impact on our mechanistic understanding of tumor suppressor genes in breast cancer, as this region has an extremely high LOH rate in human breast cancer. If our hypothesis is true, then mutational analysis of HET could become a very informative tool for breast cancer prognosis, and replacement of the non-functional HET by a wildtype HET gene might even become a gene therapy for breast cancer patients in the future.

BODY

EXPERIMENTAL METHODS AND PROCEDURES

LOH analysis

Histologic slides from archival, clinical cases were screened microscopically for adequate amounts of normal and malignant tissue. Normal tissue was obtained from each case and consisted of either adjacent benign breast tissue (terminal-duct-lobular-units, larger ducts, and stroma), skin, or lymph nodes. The corresponding malignant samples from each case included solid confluent invasive and/or metastatic breast carcinoma.

The DNA material used in our first LOH study presented in the grant proposal was isolated from manually microdissected breast tumors. Our breast cancer group has been fortunate to obtain equipment for performing laser captured microdissection (LCM). Thus, our collaborator Dr. C. Allred was able to supply us with LCM material for additional LOH studies. Briefly, single 5 μm sections were cut from the selected blocks, mounted on plain glass slides, deparaffinized, and lightly counterstained with nuclear fast red to guide laser capture microdissection (LCM) of cells from the same slides using a commercially available LCM instrument (Pixcell by Arcturus Engineering) (1; 2; 3). A transparent thermoplastic film (ethylene vinyl acetate polymer) was placed over the tissue section on the slides. Through the microscope, the operator viewed the tissue and selected clusters of target cells for harvesting. A carbon dioxide laser directed through the microscopes optics was then activated, causing the thermoplastic film to melt and fuse with the underlying targeted cells. The selected cells remained adherent to the film when it was removed from the slide, leaving the unselected tissue behind. An average of approximately 1,000 cells (about 100 cell clusters of 10 cells each) were harvested from each tissue sample.

DNA was liberated from samples by a modification of the method of Wright and Manos (4). Briefly, paraffin and lipids were first extracted by adding 0.4 ml of octane to 1.5 ml microcentrifuge tubes containing the samples. Cell debris was then digested for 3 hours at RT with 50 μl of 10mM Tris-HCl (pH 8.5), 1mM EDTA, 0.045% NP-40, and 0.045% Tween-20 containing 1.0 mg/ml proteinase K, followed by heat inactivation. Samples were independently

evaluated for LOH using the microsatellite marker D19S216 (Genethon marker AFM164zb8a: TCTTGTCACTCTAACTCCGC, and AFM164zb8m: GGCCCATGTCTTTTTTAGGT; Heterozygozity of 76%.). In addition to D19S216 we used D19S883, D19S591, D19S413 which are in close proximity on chromosome 19p13 (see below). The antisense primer was 5'-labeled, and PCR assays were performed in a total volume of 15 µl containing 1.5 mM $MgCl_2$, 1 mM spermidine base, 0.75 U Taq polymerase, 100 µM each dNTP, 100 nM primer, and 5 µl of tissue lysate diluted 1:15. Three µl of denatured DNA from each sample were loaded onto 7% polyacrylamide gels containing 32% formamide and 34% urea, and fractionated over 2.5 hours at 60 W. Gels were then transferred onto Whatman 3MM paper, covered with plastic wrap, equilibrated in a 20% methanol-20% acetic acid solution, and dried at 80° C. The intensity ratio of the two allelic bands of normal DNA relative to DNA from lesions in the same case was obtained from digitized data collected with a phosphorimager and analyzed with ImageQuant software. A conservative ratio of = 1.4 was used to define LOH in this study.

Polymerase chain reaction (PCR), Single strand chain polymorphism (SSCP), and Sequencing

Our PCR reactions are carried out on a DNA Thermal Cycler 480 (Perkin Elmer). PCR reactions for SSCP analysis are carried out using RT-PCR products from different breast cancer cell lines. We followed the protocol by Orita et al (5) with minor changes: PCR reactions are performed in 30 µl volume including 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 µM of each dNTP, 0.13 µM of each primer, 1.3-1.5 mM $MgCl_2$ and 0.5 µl Taq polymerase (PE). 5 µl of 1:24 diluted ^{32}P-dCTP will be used for incorporation of radiolabeled dNTP. The reactions are denatured at 94°C for 2 min, followed by 30-34 repetitive steps of 1 min at 94°C, 45 " at 55-60°C, 1 min at 72°C, and the last extension step at 72°C for 5 min. 1 µl of the PCR reaction will be denatured in 9 µl denaturing solution (95% deionized formamide, 10 mM NaOH, 0.25% xylene cyanol, 0.25% bromphenol blue) at 95°C for 4 min, chilled on ice for 2 min, and 4 µl are loaded onto a non-denaturing MDE (AT Biochem) gel. Gels are run at two different conditions to maximize our sensitivity for detection: at 4°C without glycerol at 40 W for 4-5 h or at room temperature with 5% glycerol at 15 W overnight. The gels will be transferred to whatman paper, dried, and exposed to X-ray film. Bands will altered mobility will be cut out from the gel, cloned and sequenced.

For the PCR of the HET exons from LCM microdissected material we use genomic DNA in a 15 µl reaction using 0.1 nmol of each primer, 100 µM of each dNTP, 3 mM MgCl2, 1 mM spermidine, and 0.5 U Platinum Taq polymerase (GIBCO). The single band PCR products are purified using QantumPrep PCR Kleen Spin Columns (BioRad).

At the beginning of the project we performed the sequencing reactions in our lab utilizing a kit from USB, following the manufacturer's protocol. Due to the increasing number of samples to be sequenced we decided to use the sequencing core at Baylor College of Medicine/ Department of Cell Biology/Dr. Lawrence Chan for future sequencing studies.

RESULTS

Task1: Month 1-10	Locate polymorphic markers with respect to HET on 19p13
Task2: Month 10-18	Perform LOH study on 170 breast tumor specimen and ten breast cancer cell lines with most appropriate marker identified in Task 1, and determine associations between LOH status and clinical and biological data as well as patient outcome
Task 3: Month 14-16	Optimize SSCP analysis on DNA from LOH-positive breast cancer cell lines

1. The LOH rate at D19S216 is extremely high in breast tumors (Task 1 and 2)

In order to confirm our previous results showing extremely high LOH on chromosome 19p13, and to more precisely determine the LOH rate at the HET locus in respect to other makers in this area, we performed an LOH study using four highly polymorphic microsatellite markers in close proximity to HET. We study was successfully performed, and the corresponding manuscript is currently under review at The Journal of the National Cancer Institute (JNCI). We submitted the manuscript under the category "Accelerated Discovery" which requires the manuscript to contain findings with very high impact for the scientific community. The Editor accepted it as "Accelerated Discovery" (only nine manuscripts have ever been accepted as "Accelerated Discovery"), and it is now being peer-reviewed. The results are described in detail in the attached manuscript. Briefly, we found that the marker colocalizing with HET/SAF-B

(D19S216) displays extremely high rates of LOH, whereas the LOH rates at markers proximal and distal to D19S216 start to decline. Thus, this finding supports our original hypothesis of HET/SAF-B being a potential tumor suppressor gene in human breast cancer.

2. Genomic DNA from LCM microdissected material can be amplified by PCR and used for sequencing

Our next goal is to test the LOH positive samples for mutations of HET in the remaining allele. Thus, genomic DNA from the LCM microdissected samples has to be amplified and sequenced. First we designed a series of primer pairs to specifically amplify the exons (Table 1). The majority of these primers are located over exon/intron boundaries. At this moment we are optimizing the PCR conditions using DNA from non-informative samples for the listed primer pairs, and we have already successfully amplified some of the products.

Table1: Primer pairs designed to amplify exons 1-9 of HET/SAF-B.

FORWARD PRIMER	POSITION	PRODUCT	REVERSE PRIMER	POSITION
1F: ATGGCGAGAGGACGGACT	961-987	271	1R:GCGTCTGGTCTAAAACTGAGAAA	INTRON
2F:GCTGCTTCCTTGTGGAGTTG	INTRON(1)	123	2R:GTGTTCGCGAGGTGACTA	INTRON (2)
3F:GGTCGCAGCAGTTGTGGTAG	1251-1270	265	3R:CTTGGAAGAAACGCCATCAC	INTRON(3)
4F:TTGATTCTCTTTTTCAGGCCA	INTRON(3)	140	4R:CCTGCCCCTTTAATTTAGCC	INTRON(4)
5F:CCAGCCAACATGTCTGTTTTT	INTRON(4)	255	5R:TCTCTCTTTGGACCCGGA	1791-1808
6F:TTGAAAGTGCTTGGAGGACAGG	INTRON(5)	497	6R:GGCAGCATGGATCTGCTTA	INTRON(6)
7F:AGATGATGCCTATTGGCCG	2210-2229	435	7R:CCCCAGCACATCCTGGTC	INTRON(7)
8F:CCTGTGTGAAAGCACGTCTG	INTRON(7)	134	8R:CAAAGCAGGGGAAGGGTG	INTRON(8)
9F:CAAACCAATGTGAATTTGTTCC	INTRON(8)	300	9R:AAACCCCAAAACGAAAAACA	2927-2946
ECP19: GGAGAACTTGCAGGACATCGACAT	413-436	656	ECPG-NEW: GTGCTTTCGCGAGCTCCTCGCTA	1046-1068

QP1F:			QP1R:		
GACTCTGTCAGGCCTAGGTGATTC	62-85	401	CCTATAGTCACACAACCTACTTCG	437-463	
QP1F:			QP2R:		
GACTCTGTCAGGCCTAGGTGATTC	62-85	535	GACACAATCTGCAACGCTTCC	577-597	

3. HET has two possible mutations in MDA-MB-468 breast cancer cell line, which can be detected by SSCP and sequencing (Task 3)

To establish the techniques before using the precious microdissected material we applied out mutation detection techniques to breast cancer cell lines. We amplified the full length HET by RT-PCR, and sequenced the PCR product in a normal breast cell line (HMEC), and in a number of breast cancer cell lines, including MDA-MB-231, MDA-MB-468, MCF-7 and T47D. Consistently we detected two changes in MDA-MB-468 (position 190, CGG/CAG, Arg/Gln and position 265, AAT/GAT, Asn/Asp). These finding were confirmed by SSCP analysis (Fig.1), where we detected altered mobilities of PCR products in MDA-MB-468 cells using primers spanning the region 153 - 409 bp (SSCP1F: 5'-tgcgagtgatcgatctgc-3', SSCP1R: 5'-ctggtctcaacatcctcctgtcca-3') and the region 206 - 356 bp (SSCP2F: 5'-ggcaacaagagcgttttga-3', SSCP2R: 5'-ttcccacaccctcttcttctgg-3). Thus, we were able to find a two nucleotide changes resulting in amino acid changes, possibly representing mutations.

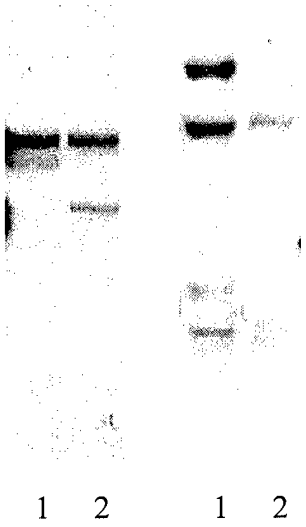

Fig.: SSCP analysis using cDNA from T47D (lane 1) and MDA-MB-468 (lane 2) breast cancer cell lines. Left panel: Primer pair SSCP1F/SSCP1R. Right Panel) Primer Pair SSCP2F/SSCP2R.

Thus, we have successfully performed and partially finished Tasks 1, 2, and 3, which were outlined for month 1-10, 10-18, and 14-16. We feel that we accomplished our research goals associated with the tasks for the first 12 month.

DISCUSSION

In twelve months since the start of the funding we have made significant progress. Fortunately, we have not encountered any technical problems yet with our studies. We have established a number of techniques needed to perform our Specific Aims, such as PCR and sequencing using genomic DNA from LCM microdissected material. Furthermore, LOH analysis using additional markers on chromosome 19p13 confirmed our original findings, and indeed made it even more likely that HET is the tumor suppressor gene at this locus (manuscript under review at JNCI). Consistent with this observation we detected base substitutions in HET in a breast cancer cell line, possibly representing missense mutations. Ongoing projects include multicolor FISH analysis with the ultimate goal to identify a marker precisely colocalizing with HET. We have also started to sequence the remaining allele from the LOH breast tumor samples.

The relocation of this project to Baylor College of Medicine Breast Center should not negatively influence the performance of the project. The Breast Center is fully equipped with the necessary resources to complete the project, and other investigators involved in the project (Dr's. Allred and O'Connell) also moved their laboratories to BCM Breast Center.

KEY RESEARCH ACCOMPLISHMENTS

- The HET locus on chromosome 19p13 displays very high LOH in human breast cancer, and other markers up-and downstream from this locus displays lower rates as compared to the HET locus.
- We have begun to optimize SSCP techniques to detect mutations.
- We have detected nucleotide changes in HET in a breast cancer cell line.

REPORTABLE OUTCOMES

Publications:

1. Loss of Heterozygozity at the HET/SAF-B Locus on Chromosome 19p13 in Human Breast Cancer. Steffi Oesterreich, D. Craig Allred, Syed Mohsin, Adrian V. Lee, C. Kent Osborne, Peter O'Connell· Submitted to JNCI (see appendix)

2. Estrogen Receptor bond to the antiestrogen tamoxifen strongly interacts with the nuclear matrix protein HET/SAF-B, a novel inhibitor of estrogen receptor-mediated transactivation. In preparation.

Abstracts:

1. Estrogen Receptor Bound to the Antiestrogen Tamoxifen Strongly Interacts with the Nuclear Matrix Protein HET/SAF-B, a Novel Inhibitor of Estrogen Receptor-Mediated Transactivation Steffi Oesterreich, Qingping Zhang, Torsten. Hopp, Suzanne A. W. Fuqua, Marten Michaelis, Holly H. Zhao, Jim R. Davie, C. Kent Osborne, and Adrian V. Lee. Breast Cancer Research and Treatment, in press (to be presented at a Oral Presentation at the San Antonio Breast Cancer Symposium December 1999)

Presentation:

7th SPORE Investigators Workshop, July 11-13, 1999 Washington DC. Steffi Oesterreich, Adrian V. Lee, Peter O'Connell, C.Kent Osborne: HET/SAF-B, a Nuclear Matrix Protein which Functions as an ER-Corepressor and Resides at a Chromosomal Locus Frequently Lost in Human Breast Cancer.

Invited Speaker at International Meeting of Flemish Gynecological Oncology Group, Brussels, Belgium, December 3-4, 1999

Employment opportunity:

I accepted a new position as an Assistant Professor at Baylor College of Medicine (BCM). The College is one of the very most competitive places in the USA, and there is no doubt in my mind that the funding of my project "HET is a potential new tumor suppressor gene in human breast cancer" supported this great new employment opportunity for me.

CONCLUSIONS

There are no major modifications to the experimental plans. The major change will be relocation of the principal investigator's laboratory, but I strongly believe that the project will be strengthened by the unique resources at Baylor and by the surrounding of additional outstanding clinicians and scientists. The scientific breadth at Baylor will add significantly to my scientific productivity.

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APPENDICES

1. Manuscript submitted to JNCI

(Loss of Heterozygozity at the HET/SAF-B Locus on Chromosome 19p13 in Human Breast Cancer. Steffi Oesterreich, D. Craig Allred, Syed Mohsin, Adrian V. Lee, C. Kent Osborne, Peter O'Connell)

2. Abstract, Breast Cancer Research and Treatment

(Estrogen Receptor Bound to the Antiestrogen Tamoxifen Strongly Interacts with the Nuclear Matrix Protein HET/SAF-B, a Novel Inhibitor of Estrogen Receptor-Mediated Transactivation Steffi Oesterreich, Qingping Zhang, Torsten. Hopp, Suzanne A. W. Fuqua, Marten Michaelis, Holly H. Zhao, Jim R. Davie, C. Kent Osborne, and Adrian V. Lee)

Loss of Heterozygozity at the HET/SAF-B locus (19p13) in Human Breast Cancer

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Keywords: loss of heterozygozity, chromosome 19p13, breast cancer, HET/SAF-B, tumor suppressor gene

Abbreviations: LOH, loss of heterozygozity; HET/SAF-B, hsp27-ERE-TATA-binding protein/scaffold attachment factor B.

Supported by a Howard Temin Award (K01 CA77674) and a US Army grant DAMD17-98-1-8340 to S.O., and a Breast Cancer SPORE (PHS P50 CA58183) and a P01 CA30195.

Submitted to Journal of the National Cancer Institute as Accelerated Discovery

Abstract

We have recently discovered that the nuclear protein HET/SAF-B is an ER corepressor. Because it has become clear that many steroid receptor cofactors play important roles in breast tumorigenesis, we asked whether HET/SAF-B could also be involved in breast cancer. To address this question, we performed loss of heterozygozity (LOH) studies with a microsatellite marker (D19S216) colocalizing with HET/SAF-B on chromosome 19p13.2-3. Using laser capture microdissected material, we detected extremely high LOH rates (78.4%) for this region. With additional microsatellite markers up- and downstream from D19S216, we found the peak LOH rate at the marker colocalizing with HET/SAF-B. The finding of very high LOH at this locus strongly indicates the presence of a breast tumor suppressor, which could be the ER corepressor HET/SAF-B.

Introduction

The estrogen receptor (ER) is a nuclear steroid receptor that upon activation by its ligands initiates a cascade of events resulting in increased cellular proliferation in its target tissues (1). Since estrogen is one of the most potent mitogens for breast cancer cells, it is no surprise that ER is the most important target for endocrine therapy of breast cancer (2). Recently, a number of factors which regulate nuclear hormone receptor activity have been identified. Cofactors capable of increasing receptor action, termed coactivators, include TIF1, RIP140, SRC-1/NcoA1, TIF2/GRIP1, pCIP/RAC3/AIB1/ACTR/TRAM-1, and CBP/p300 (3; 4). The family of corepressors is smaller; the best characterized being nuclear receptor corepressor (N-CoR) (4; 5) and the silencing mediator of retinoid and thyroid receptors (SMRT) (6; 7). The overexpression of coactivators or the loss of corepressors could lead to unregulated estrogen-dependent pathways related to mammary epithelial proliferation, and thus to breast tumorigenesis. And indeed, some of the ER cofactors have recently

been characterized as playing major roles in breast tumorigenesis. The ER coactivator AIB1 was cloned during a search on the long arm of chromosome 20 for genes whose expression and copy number are elevated in human breast cancer, and subsequent analysis in 105 breast tumor specimens confirmed its overexpression (8). Interestingly, the tumor suppressor gene BRCA1 has recently been characterized as an ER corepressor (9), again suggesting that ER coregulators are crucial in breast tumorigenesis. Thus, it is expected that other ER coactivators and corepressors might play similar important roles in breast cancer development and progression.

We have recently discovered that the nuclear matrix protein HET/SAF-B (10; 11) is an ER corepressor (12). ER and HET/SAF-B interact in *in vitro* binding assays (GST-pulldown assays) and in cell lines (co-immunoprecipitation experiments). In cell lines, there is binding of HET/SAF-B to ER in the presence or absence of estradiol; however, binding is significantly increased by the antiestrogen Tamoxifen. Overexpression of HET/SAF-B results in repression of estrogen-mediated transactivation by the ER. Furthermore, as a result of HET/SAF-B overexpression, the antagonist activity of tamoxifen on ER can be enhanced, and the agonist activity of tamoxifen can be inhibited.

These results led us to investigate whether the ER corepressor HET/SAF-B could also be involved in breast tumorigenesis. Towards this goal we analyzed whether the chromosomal locus for HET/SAF-B is a frequent target for chromosomal aberrations, i.e. allelic deletion. Allelic deletion manifested as loss of heterozygozity (LOH) at polymorphic loci is recognized as a hallmark for genes involved in tumor suppression; thus, high LOH at the HET/SAF-B locus would suggest that this recently identified ER cofactor could play an important role in breast tumor suppression. And indeed, in the present study we have found extremely high rates of LOH (78%) at the HET/SAF-B locus on chromosome 19p13 in human breast cancer specimens.

Methods

Microdissection: The 57 cases comprising this study were paraffin-embedded archival primary breast cancers. For 52 of the cases, a single paraffin section yielded sufficient normal tissue (terminal duct lobular unit) and primary cancer. For five of the cases, normal lymph node tissues were recovered from separate blocks. Single 5 μm sections were cut from the selected blocks, mounted on plain glass slides, deparaffinized, and lightly counterstained with nuclear fast red to guide laser capture microdissection (LCM) of cells from the same slides using a commercially available LCM instrument (Pixcell by Arcturus Engineering) (13; 14). Briefly, a transparent thermoplastic film (ethylene vinyl acetate polymer) was placed over the tissue section on the slides. Through the microscope, the operator viewed the tissue and selected clusters of target cells for harvesting. A carbon dioxide laser directed through the microscope optics was then activated, causing the thermoplastic film to melt and fuse with the underlying targeted cells. The selected cells remained adherent to the film when it was removed from the slide, leaving the unselected tissue behind. An average of approximately 1,000 cells (about 100 cell clusters of 10 cells each) were harvested from each tissue sample. All cases were obtained with IRB approval from the patient archives at University Hospital, University of Texas Health Science Center at San Antonio.

LOH analysis: LOH analysis was performed as recently described (15). Briefly, DNA was liberated from samples by a modification of the method of Wright and Manos (16). The embedded cells were digested for 18-20 hours at 37° C in 60 μl of 10mM Tris-HCl (pH 8.5), 1mM EDTA, 0.045% NP-40, and 0.045% Tween-20, containing 1.0 mg/ml proteinase K. The protease was then inactivated at 95°C for 10 minutes. PCR assays were performed in a total volume of 15 μl containing 1.5 mM $MgCl_2$, 1 mM spermidine base, 0.75 U Platinum Taq DNA Polymerase (Gibco), 100 μM of each dNTP, 100 nM primer, and 5 μl of tissue lysate diluted 1:2. Samples were evaluated for LOH using the microsatellite

markers D19S216, D19S413, D19S591, and D19S883. The primer pairs were obtained from Research Genetics, Inc. (Birmingham, AL). Mapping data were obtained from the Genome Data Base (GDB™) at Johns Hopkins University (17; 18). The antisense primer was 5'-labeled with γ-^{32}P-ATP and polynucleotide kinase. Three µl of denatured DNA from each sample were loaded onto 7% polyacrylamide gels containing 32% formamide and 34% urea, and fractionated over 2.5 hours at 60 W. Gels were then transferred onto Whatman 3M paper, covered with plastic wrap, equilibrated in a 20% methanol-20% acetic acid solution, and dried at 80° C.

The intensity ratios of marker alleles from paired normal and breast cancer tissues were calculated from digitized data collected with a storage phosphor device and analyzed with the Molecular Dynamics ImageQuant software package (Molecular Dynamics, Sunnyvale, CA). LOH was considered positive when the proportion [(tumor allele 1/tumor allele 2)/(normal allele 1/normal allele 2)], equaled either less than 0.71 (tumor allele 1 LOH) or greater than 1.4 (tumor allele 2 LOH).

Results and Discussion

We recently assigned HET/SAF-B to chromosome 19-band p13.2-13.3, by fluorescent *in situ* hybridization (19). On the chromosome 19 radiation hybrid map (20) HET/SAF-B is positioned at 34.7 cRays. The polymorphic marker D19S216 has been placed at 35.9 cRays on the same map, so that HET-SAF-B maps in the D19S591-D19S216 interval just proximal to D19S216. We tested this region for LOH using D19S216 and a series of additional markers spanning band 19p13, namely D19S884 (5.5 cM), D19S591 (9.8 cM), D19S216 (20.0 cM), and D19S413 (31.2 cM). LOH studies were carried out using normal and primary breast cancer tissues from 57 patients. Three of the specimens showed evidence of microsatellite instability and were excluded from further analysis.

The results of this LOH study are shown in Table 1. Marker D19S216 near HET/SAF-B shows the highest rates of LOH (78%). Figure 1 summarizes data from the subset of 25 D19S216-informative cases with interstitial LOH events. An additional 12 cases (not presented) either showed no LOH, or showed LOH for all markers. These breakpoints can map the smallest region of overlap for the LOH region(s). The majority of the cases show LOH events spanning D19S591-D19S216. Four cases (96, 179, 1086, and 1094) showed LOH events but remained heterozygous for D19S216. LOH events in four other cases (190, 207, 613, and 742) lost only D19S216, and case 810 lost DNA sequences including D19S216 and D19S413. No D19S216-informative tumors exclusively lost D19S413. These data indicate that the interval between D19S591-D19S216 harbors a tumor suppressor gene important in human breast cancer. As mentioned above, HET/SAF-B maps to this interval.

Kerangueven et al (21) had identified D19S216 as a marker with consistent loss (20-30%) in breast cancer using genomic DNA isolated from whole breast tumors. Bignell et al. (22) also performed an LOH study on chromosome 19p13.3, with the goal of analyzing chromosomal loss of the LKB1 gene. (The serine/threonine kinase LKB1 is mutated in patients with Peutz-Jeghers Syndrome, resulting in intestinal hamartomas associated with an elevated risk for cancer). They used the LKB-linked marker D19S565, which co-localizes with D19S883 (see Fig. 1). The Bignell study detected LOH in 7.5% of informative breast cancer specimens, as compared to 21.6% in our study.

It is difficult to compare the LOH rates from our present LCM-based study to those of previous reports, since only a few studies using LCM material have been published. For instance Bignell et al saw 7.5% (3/40) LOH with D19S565 using whole tissue genomic DNA while we found 21.6% (8/37) using LCM material. Though part of this difference might simply reflect the small number of samples, we have previously seen that LCM enriches for tumor cells and thus always results in a higher LOH

rate. As an example, we found 53% LOH (32/60) at D19S216 using manually microdissected tissue (data not shown), but saw 78% LOH (29/37) using LCM. Brown et al. (23) also noted elevated LOH rates at 8p12-22 in ovarian cancers when comparing LCM-based LOH rates to those determined in previous allelotyping studies. Tamura et al. (24) noted 35% LOH at the RB locus on chromosome 13 from whole tumors, but a 59% rate of RB locus LOH when the tumor cells were enriched by flow sorting. We have also determined a 56% rate of LOH at the RB locus (data not shown) in our LCM-based breast cancer studies.

Our rationale for this study was that the ER corepressor HET/SAF-B might represent a new tumor suppressor gene, and our present finding would certainly support this hypothesis. LOH frequency at D19S591--HET/SAF-B—D19S216 region is among the highest yet measured in breast cancer. Our own unpublished western blot analyses have also demonstrated variations in the abundance of HET/SAF-B in breast tumor specimens, and in 16% of the tumors (10 out of 61) no protein was detectable even after prolonged exposure of the film. In addition, in two of these cases we detected apparently truncated versions of this protein. Certainly the estrogen receptor corepressor HET/SAF-B is a candidate for the tumor suppressor at this locus, but mutation analyses will be required to precisely define the gene(s) at 19.13.3 causatively involved in breast tumorigenesis.

Table I. LOH Frequencies for Chromosome 19p13.3 Markers

Marker	Location (cM)	Observed Heterozygosity	LOH Frequency
D19S883	5.5	37/54 (68.6%)	8/37 (21.6%)
D19S591	9.8	36/54 (66.7%)	17/36 (47.2%)
D19S216	20.1	37/54 (68.5%)	29/37 (78.4%)
D19S413	31.2	35/54 (64.8%)	11/35 (31.4%)

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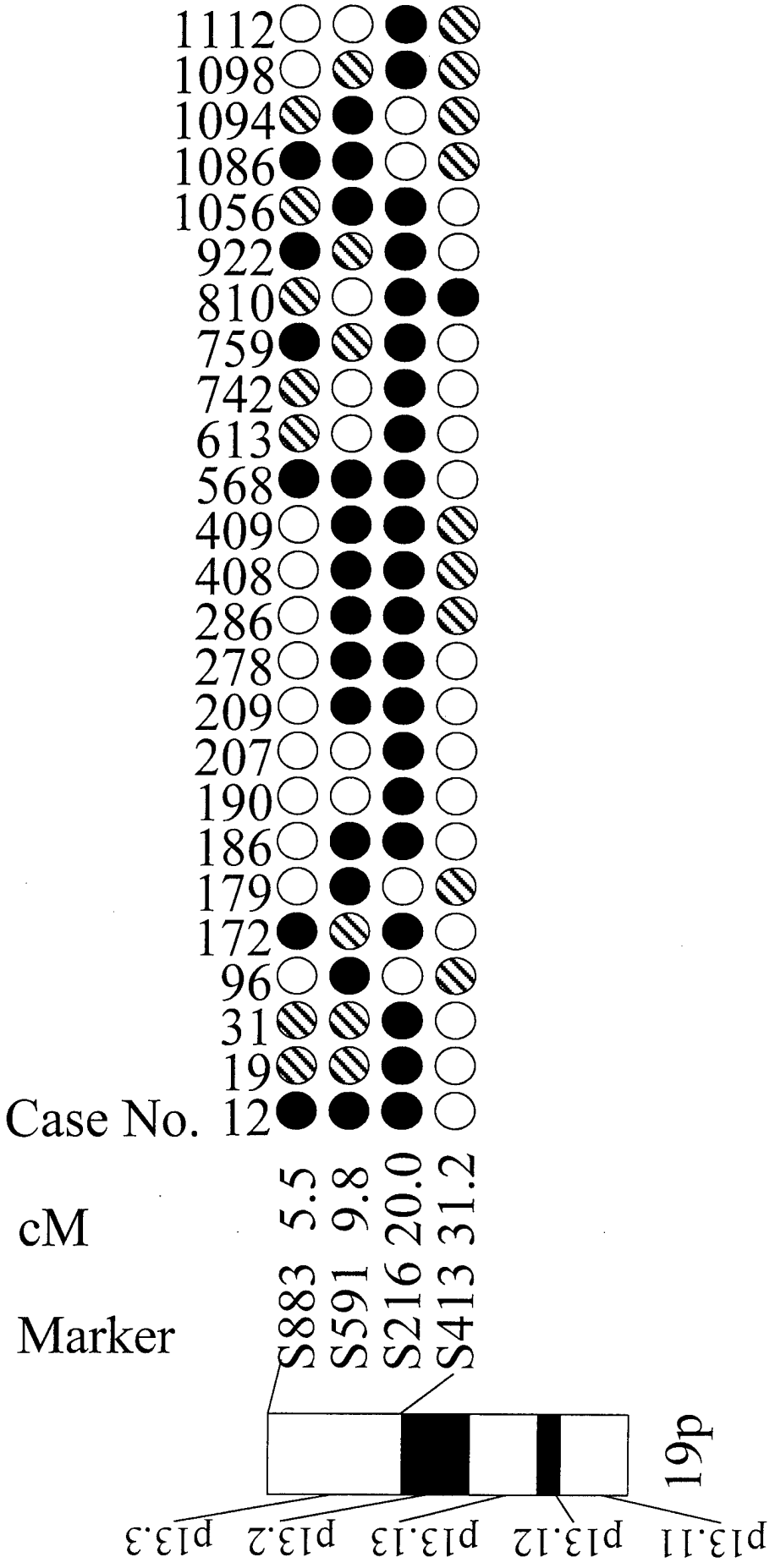
Figure Legend:.

Fig. 1: LOH profile in the D19S216-HET/SAF-B region. Left: An idiogram of chromosome 19p detailing the region of interest. Right: The LOH profile 25 cases informative for D19S216 with interstitial breakpoints. Breast cancer cases 12-1112 are displayed vertically. Marker data is presented horizontally: filled circles denote cases with LOH, open circles denote heterozygous cases (no LOH), and hatched circles show non-informative cases.

FIGURE 1


1112
1098
1094
1086
1056
922
810
759
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613
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409
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278
209
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190
186
179
172
96
31
19
12
Case No.
cM
5.5
9.8
20.0
31.2
Marker
S883
S591
S216
S413
19p
p13.3
p13.2
p13.13
p13.12
p13.11

Estrogen Receptor Bound to the Antiestrogen Tamoxifen Strongly Interacts with the Nuclear Matrix Protein HET/SAF-B, a Novel Inhibitor of Estrogen Receptor-Mediated Transactivation

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The estrogen receptor (ER) is a ligand-dependent transcription factor that acts in a cell and promoter specific manner. Evidence suggests that the activity of the ER can be regulated by a number of other stimuli (e.g. growth factors) and that the effects of the ER are modulated by nuclear factors termed coregulators. While the interplay between these factors may in part explain the pleiotropic effects elicited by the ER, there are several other less well described mechanisms of control, such as interactions with the nuclear matrix. Here we report that the nuclear matrix protein/scaffold attachment factor HET/SAF-B is an ER-interacting protein. ER and HET/SAF-B interact in *in vitro* binding assays (GST-pulldown assays) and in cell lines (co-immunoprecipitation experiments). In cell lines, there is association between ER and HET/SAF-B in the presence or absence of estradiol, however binding is significantly increased by the antiestrogen tamoxifen. Overexpression of HET/SAF-B results in repression of estrogen-mediated transactivation by the ER. Furthermore, as a result of HET/SAF-B overexpression, the antagonist activity of tamoxifen on ER can be enhanced, and the agonist activity of tamoxifen can be inhibited. While the identity of high-affinity binding sites for the ER in the nuclear matrix has been known for a long time, we provide the first evidence of a specific nuclear matrix protein binding to the ER. Furthermore, our data showing that HET/SAF-B strongly binds to ER in the presence of tamoxifen suggests that it may be important for the antiestrogenic effect of tamoxifen.

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